Molecular characterization and *in silico* studies of *cowpea aphid borne mosaic virus* and *papaya ringspot virus*

A
RESEARCH PLAN
SUBMITTED

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The RNA genome contains one long open reading frame (ORF) expressed as a 350kDa polyprotein precursor (Riechmann *et al.*, 1995). This is proteolytically processed by viral and host proteases (Langenberg and Zhang, 1997) into seven smaller proteins denoted as P1, helper component (HC), P3, cylindrical inclusion (CI), nuclear inclusion A (NIa), nuclear inclusion B (NIb), capsid protein (CP), as well as two small putative proteins known as 6K1 and 6K2 (Riechmann *et al.*, 1992). Figure 1 shows the representative diagram of potyvirus genome.

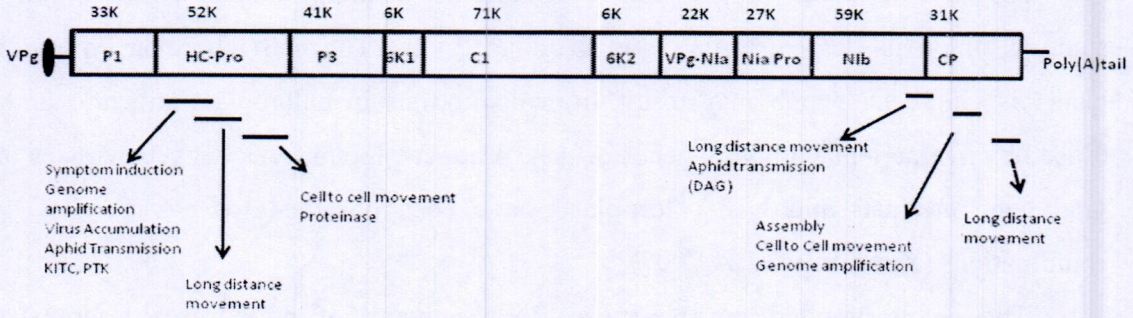

Figure 1: Monopartite potyviral genome displaying functional products after translation and cleavage. The size (in Kilodalton,K)of each protein is noted above.

Plant viruses are mainly transmitted through vectors from one crop to another crop and during the transmission process vectors play important role. Multiple number of vectors like aphids spread potyvirus and most are from the subfamily *Aphidinae* (genera *Macrosiphum* and *Myzus*). In case of potyvirus there are multiple genes are responsible for infection, in which two main genes are responsible for transmission through vector, the coat protein (CP) and its derivatives (read through CP and minor CP) and the nonstructural protein, such as helper component (HC) protein. *Myzus persicae* (green peach aphid), is the most common aphid species and causal agent for the transmission of potyviruses in plants. Transmission of virus may occur in two modes: non-persistent and persistent mode. All potyviruses are mainly transmitted through nonpersistent mode. 'Bridge' hypothesis (Guo Bihong *et al.*, 2011) also involved in transmission, where the HC binds to aphid mouthparts on one side and virions on the other side. Highly conserved three amino acid motif, asp-ala-gly (DAG), located near the N terminus of the coat protein (CP), is important for aphid transmission. Some scientists prove that HcPro acts coordinately with the coat protein (CP) for several of these functions: aphid transmission and also, less expectedly, virus movement.

Hc-Pro is especially important in this respect because it is a multifunctional protein involved in a number of processes of the virus cycle. HcPro acts coordinately with the CP for several of those functions: aphid transmission (Roudet-Tavert *et al.*, 2002) and also, less expectedly, virus movement (Lee & Lucas 2001). This opens the possibility that these proteins engage in a direct interaction. There are many experimental exposures involved, which show the interaction between HCpro and CP viral proteins.

Potyviruses transmission can occur mechanically through tools, by insect vectors associated with the crop, especially aphids in a non-persistent manner (Martinez *et al.*, 2010) or even by seed (Diaz *et al.*, 2010; Alvarez *et al.*, 2011).

Polymerase chain reaction (PCR) is an extremely sensitive and specific technique for the detection and identification of plant pathogens. C.Ha *et al.* (2008) used degenerate primers for PCR to confirm the presence of Potyviruses in the leaves of naturally infected symptomatic crop plants.

Conformational information on the secondary structures of a protein, like the order of its local alpha helix or beta sheet elements, or the tertiary (3D) structure showing the organization of those elements, may provide clues about the protein's locations, interactions and functions and assist in the distinction of its functional or structural domains. If experimentally determined structures are not available, structural elements and 3D models of poorly known proteins can be predicted *in silico*.

Recombination among the HC-Pro sequences was analyzed using the RDP, GENECONV, CHIMAERA, MAXIMUM CHISQUARE, BOOTSCAN and SISCAN methods implemented in the RDP3 vb41 program (Martin *et al.*, 2010).

Tracking virus movement and localization will allow better understanding of the soybean-SMV association at the cellular and sub-cellular levels. It may also show which genes or structures are involved in the virus-host interaction, viral movement, and subsequently resistance. Different methods have been used to track virus movement in plants in conjunction with studies to characterize movement proteins and resistance mechanisms. Tissue print hybridization with digoxigenin-labeled RNA probes has been useful in studying the movement of viruses through the vasculature of the plant (Mas and Pallas, 1995). In-situ hybridization is a similar detection method whereby fluorescent or digoxigenin-labeled nucleic acid probes are

Objectives:

1. Molecular characterization of Indian isolate of *Cowpea aphid borne mosaic virus* isolates RR4, RR3 and *Papaya ringspot virus* isolate R3.
2. *In silico* analysis of genome of two Indian isolates of *Cowpea aphid-borne mosaic virus* isolates RR4, RR3 and *Papaya ringspot virus* isolate R3.
3. Coat protein-mediated resistance against an Indian isolate of the Cowpea aphid-borne mosaic virus RR3 in *Nicotiana tabacum* L.
4. Transmission and transient gene expression studies of *Cowpea aphid borne mosaic virus* and *Papaya ringspot virus*.

3. Molecular characterization of complete genome of *cowpea aphid borne mosaic virus* and *papaya ringspot virus*

The whole genome sequence of CABMV was determined by seven different degenerate primers. The primers will design on the basis of multiple sequence alignment of whole genome sequences which are available in NCBI data bank including CABMV-Z, CABMV-BR1 and some primers will obtained from previously reported primer sequences.

Whole genome of PRSV was determined from three different pairs of primers. We used Primer3 software for designing the primers sets.

Cloning and Sequencing of PCR product

Seven amplified PCR products of CABMV and three of PRSV were purified using the QIAquick PCR Purification Kit (Qiagen) and then cloned into the pGEMT Easy Vector 2 (Promega) following the manufacturers' instructions. Plasmid DNA was extracted from white colonies using the small-scale alkaline lysismethod. Inserts were confirmed by restriction enzyme analysis and sequencing.

4. Sequence analysis of *cowpea aphid borne mosaic virus* and *papaya ringspot virus* genome

Sequence analysis included the BLAST , Sequence similarity index, Phylogenetic analysis as well as Recombination detection programme. Whole genome sequences were initially selected for homology using the BLAST program (http:// www.ncbi.nlm.nih.gov/BLAST) and the sequences showed better scores selected for genetic variability study. The sequence similarity matrix between amino acid sequences and nucleotide sequences was calculated using the BioEdit software version 5.09.04. Amino acid sequences were aligned using Clustal W (10) and Neighbor-joining tree was generated using MEGA v. 5.2 programs.

Recombination of sequences will be analyze by using the RDP, GENECONV, CHIMAERA, MAXIMUM CHISQUARE, BOOTSCAN and SISCAN methods implemented in the RDP3 vb41 program (Martin *et al.*, 2010). To determine the site-specific selection pressures acting on the HC-Pro gene, the ML codon-substitution model implemented in the CODEML program of the PAML4 package (Yang, 2007) and four different codon-based maximum-likelihood methods

cultured in LB broth supplemented with gentamycin, rifampacin and kanamycin, for overnight at 28^0C, 200rpm. Cells were harvested by centrifugation (15 min at 5000rpm), washed twice with ice-cold water and infiltered medium (10 mM MES pH 6.3, 10 mM $MgCl_2$ and 150 µM acetosyringone) to obtain a final volume absorbance of 0.5 at 600 nm. The bacterial suspension was incubated at room temperature for 2-3 hours before agro-infiltration. Further, suspension culture (pBI121CP CABMV and pBI121 CP PRSV) will be forced on to lower surface of *N. benthamiana* leaves of 15 test and 5 control plants using a blunt-tipped 1ml plastic syringe. Both test and control plants were kept in a glasshouse for 2 to 3 days at 25°C under a 16/8 h light/dark cycle.

7. PCR amplification and GUS histochemical staining

Total DNA was extracted from all agroinfiltered plant and control plant using CTAB methods. PCR was then performed by using CP specific primers. Further positive tests and control plants samples were taken for GUS histochemical staining.

Histochemical analyses of GUS expression were carried out in test and control plants. Plant samples were fixed in a fixation solution containing 50 mM sodium phosphate buffer (pH 7.0), 0.3% formaldehyde, and 0.3 M mannitol. They were then washed three times with 50 mM sodium phosphate buffer (pH 7.0), and soaked at 37^0C for 24 h in the GUS assay solution, which included 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA (pH 8.0), 2 mM 5-bromo-4-chloro-3-indolylh- d-glucuronide, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 0.1% (v/v) Triton X-100. Samples for heat treatment were also stained for 12 h. After staining, the tissues were soaked in several changes of 95% ethanol to stop enzyme reaction and remove chlorophyll [20].

8. Development of transgenic against coat protein (CP) of *Cowpea aphid borne mosaic virus*

Coat Protein gene of potyvirus will be introduced in binary vector pBI121, which have kanamycine resistance selection marker (nptII). The binary construct will be mobilized into *Agrobacterium tumefaciens* GV3031 freeze thaw method. One positive conjugant will be chosen for:

1) Agroinfilteration: for detect the movement of gene with the help of reporter gene.

2) Transformation of *N. tabacum* L. leaf discs following a procedure.

Future Prospectus

The purpose of my Phd work is to characterize the molecular nature of Potyviridae family in India and to study the pathogenicity and epidemiology of potyvirus strains. This will help us to gain a better knowledge on the genetic determinants that drive Potyvirus molecular diversity and evolution. The key aims are:

- Molecular characterization of the population structure of intercepted Potyvirus field isolates.
- Biological and further molecular characterization of all major Potyvirus isolates: mapping of the genetic determinants responsible for ringspot necrosis in potato.
- To Identify agricultural important crop varieties that displays different levels of susceptibility to Potyvirus.
- Study Potyvirus strains epidemiology (groups and subgroups) by setting up field trials to monitor virus spread and transmission during a growing season, in order to identify potential association of Potyvirus isolates with some aphid species.
- To further develop molecular diagnostic methods that will discriminate between Potyvirus variants.

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Introduction:

Now a day's agriculture loss is very critical problem and this problem is mainly generated by human being in the form of environmental pollution. Instead of human being multiple types of biotic and abiotic stress is also responsible for agricultural loss. Biotic stress includes many microorganisms like bacteria and viruses. In abiotic stress many physical factors are involved like salt stress and drought stress etc. Many types of vector present in environment which cause the transmission of potyvirus. Multiple numbers of microbial populations are involved in agricultural field, in which some population have synergistic effect and some create so much losses in agriculture. Potyvirus also play an important role in significant losses in agricultural, pastoral, horticultural and ornamental crops. Potyviruses constitute an important group of viruses causing severe economic damage to various crops; they are believed to be responsible for about 40% of all plant diseases of virus origin. Potyviruses infect plants and belong to the family *Potyviridae*. The genus is named after the type virus - Potato Virus Y (PVY). The potyvirus group is the largest of the 34 plant virus groups and families currently recognized. This group contains at least 180 definitive and possible members (30% of all known plant viruses) which cause significant losses.

A virus is considered to be a definitive member of the Potyvirus group if it: -

(1) has filamentous particles of characteristic size and properties;
(2)Is transmitted non-persistently by aphids;
(3) Induces the formation of characteristic cytoplasmic inclusions ('pinwheels'); and
(4) Is clearly serologically distinguishable from morphologically similar viruses.

Most potyviruses induce conspicuous symptoms, often causing stunting and yield losses. They usually induce longitudinal chlorotic or necrotic streaks in the leaves of monocotyledonous species, and chlorotic vein banding, mosaic mottling, necrosis, and/or distortion of leaves in dicotyledonous species. Flowers, seeds, and fruits are also affected by numerous potyviruses. Potyviruses are filamentous plant viruses whose genomes consist of a single 9.5 kb molecule of single-stranded, positive-sense RNA. Potyviral RNA contains a covalently linked protein (VPg) at the 5' terminus and is polyadenylated at the 3' terminus.

Review of literature:

Potyviruses are accounted for 40% of viral diseases of various crops including vegetables and legumes. Potyviruses can be transmitted through plant sap, seeds and many aphid species. Due to ease of spread of these viruses detection of such viruses are crucial as to the control of the incited diseases.

Depending on the combination of virus and host, as well as the environmental conditions, *Potyvirus*-infected plants exhibit vasrious symptoms, including mosaic patterns, vein banding, stunting, chlorosis, leaf distortion, and necrotic lesions. The most common symptom of such plants is chlorosis, which may result from disruptions in chloroplast structure and function caused by interactions with viral genes or gene products. Interactions between viruses or specific viral gene products and host chloroplasts have been observed for a variety of virus–host combinations (Yongsheng *et al.* 2007).

The single-stranded positive-sense RNA genome of potyviruses is translated to a polyprotein which is subsequently processed into as many as ten functional proteins (Riechmann *et al.*, 1992). The helper component-proteinase (HC-Pro) (Maia *et al.*, 1996) and coat protein (CP) (Shukla *et al.*, 1994;Mahajan *et al.*, 1996) are examples of multifunctional potyvirus proteins The N-terminal part of HC-Pro affects virulence (symptom severity), genome amplification and virus accumulation (Atreya *et al.*, 1992; Atreya & Pirone, 1993; Kasschau & Carrington, 1995). KITC and PTK amino acid motifs of HC-Pro are required for transmissibility by aphid vectors (Atreya & Pirone, 1993; Huet *et al.*, 1994).

The genus *Potyvirus* of the family *Potyviridae* represents the most important and largest genus of plant viruses. Species belonging to this genus can share many common properties and often cross react in serological tests. In the past, identification of potyviruses, based on biological and serological properties, has not provided a definite solution due to the large size of the group and the extensive biological and antigenic variation within the group. In recent years, a consensus has emerged in the identification and classification of potyviruses at the species level. This is largely based on amino acid and/or nucleotide sequence comparison of the genome, in particular the coat protein cistron (CP) and the 3_-untranslated region (3_-UTR) (Inoue-Nagata *et al.,* 2001).

used to detect viral nucleic acid in plant tissues. Petioles and stem sections can be used and the hybridization may be detected using an epifluorescence or confocal microscope. Detection of the probe (gold, antibody) is also possible using either a light or electron microscope (Troxler *et al.*, 1990).

Another approach to studying viral movement is the use of green fluorescent GFP or β-glucuronidase (GUS)-labeled infectious DNA clones of virus and monitoring virus movement by doing a time course analysis for detecting viral signals in the host tissue. Dolja *et al.* (1992) used an infectious clone of TEV containing an insertion of the bacterial GUS gene between the HC-Pro gene and the N-terminal 35 kDa protease gene. Time course analysis of GUS expression made possible the detection and visualization of virus activity in single mechanically-inoculated cells, in adjacent cells, and in phloem cells associated with systemic or long distance movement of the virus. This allowed the tracking of both cell-to-cell and long-distance movement of virus.

Its multiple functions in the infection cycle implicate HCpro as an important and probable target for the plant defence system. HCpro of several potyviruses is the determinant, or one of the determinants, of necrosis in infected plants. HCpro and its structure have been under strong selection, as mutations or insertions along HCpro reduce its silencing-suppression capacity and reduce the fitness or systemic movement of virus (Kasschau and Carrington, 2001, Torres-Barceló *et al.,* 2008). Hence, any interaction partners of HCpro are of interest as they might be potential candidates for durable resistance.

Material and Methods:

1. Survey and collection of virus infected cowpea, papaya and other crops samples

Sample collection will perform all over region of Rajasthan as well as some parts of India. According to observation symptom in crops like puckering, mottling, mosaic and stunting will collected.

2. Primary detection of potyvirus infection

Primary detection for presence of CABMV, PRSV and other potyvirus will be confirmed by universal primers (Gibbs et al. 1997) which codes for conserved region on it.

2a. RNA isolation and C-DNA preparation preparation of CABMV and PRSV

Isolation of RNA will be perform by TRIzol Reagent, is a single, homogenous solution for the isolation of total RNA. This phenol –based reagent contains a unique combination of denaturants and RNase inhibitors and is used in a convenient, single –step disruption separation procedure. The tissue or cell sample is homogenized or disrupted in the TRI Reagent, chloroform is mixed with the lysate, and the mixture is separated into three phases by centrifugation. The RNA is then precipitated from the aqueous phase with isopropanol. The entire procedure can be completed in no more than one hour to produce high yields of intact RNA.

2b. RNA will be directly used for C-DNA preparation. Steps involved in C-DNA preparation

1. RNA sample, Nuclease free water and OligodT will mixed and incubate at 65^0C for 10 minutes in Thermal cycler.
2. After 10 minute incubation tube will be frequently kept on ice.
3. Side wise prepare mixture of Amv Reverse Transcriptase, RT buffer (5X), Mgcl2, dNTPs and make volume upto 25 µl with nuclease free water.
4. This mixure will be mixed in first step tube and incubate for 42^0C for 1 hour and subsequently at 72^0C for 5mins for stop reaction.

(Concentration of Amv Reverse Transcriptase (RT) 10 units per reaction for 25 µl)

implemented on the Datamonkey server (http://www.datamonkey.org) (Delport *et al.*, 2010) were used to estimate the rate of nonsynonymous (dN) and synonymous (dS) substitutions at each codon site. DnaSP version 5.10 (Librado and Rozas, 2009) was used to analyzed the data by Tajima's D test, and Fu and Li's D and F tests.

5. Biological transmission study of *cowpea aphid borne mosaic virus* and *papaya ringspot virus*

Virus infected crops will be produce from mechanical transmission of virus infected sap of papaya and cowpea leafs and by aphids inoculation in healthy tomato, wheat, potato as well as cowpea plants. The buffer use for mechanical transmission will be composed of 0.1 M phosphate buffer, pH 7.0. Crude sap will obtain by grinding the infected young leaves of infected crops with the buffer. Mechanical inoculation will be conduct by rubbing sap using a cotton swab on the dusted leaves with Carborundum (400 meshes) of the test plants. Inoculated plants were caged in an insect proof greenhouse at 25°C. A healthy plant was used as a negative control and inoculated with the phosphate buffer only.

Apterous *Myzuspersicae* Sulzer aphids through plants will collect in winter season and keep in glass vials for 2–3 h of pre acquisition fasting. Groups of starved aphids will allow to feed during a 10 min acquisition access period through stretched parafilm membranes on a virus infected leaf samples and 20 % sucrose prepared in TSM buffer. Groups of aphids will be placed on test plants for inoculation and allowed to feed overnight before spraying with insecticide. Symptoms were recorded 10 days later by visual inspection and confirmed by RT-PCR amplification of total nucleic acids extracted from plants using Universal as well as degenerate primers against potyvirus.

6. Binary constructs preparation and Agro-infiltration

Amplified Coat protein gene of both plant virus (CABMV and PRSV) will be eluted and clone in pBI121 adjacent to GUS followed by restriction and ligation method. Selected positive clones and pBI121 vector were then transformed in *Agrobacterium tumefaciens* GV3101 by cold shock method and cultured in LB agar plates containing 100 ug/ml kanamycin, 25-50 ug/ml Gentamycin, 10 ug/ ml rifampicin for overnight at 28^0C. The positive transformed clones (pBI121CP CABMV and pBI121 CP PRSV) and pBI121as a control both separately were

The transformed leaf discs will be regenerated on selective Murashige–Skoog (MS) medium containing Gamborg B5 vitamins, antibiotics (500 mg/l cefotaxime and 100 mg/l kanamycin) and hormones (1 mg/l zeatin and 0.1 mg/l indole acetic acid [IAA]) in a growth chamber. Shoots regenerating from the explants were subsequently transferred to MS medium containing 0.1 mg/l each of zeatin and IAA for shoot elongation and, finally, to a medium containing antibiotics, indole butyric acid [IBA] 0.05 mg/l but no cytokinin for root formation. The putative transgenic plants will be finally planted in pots and grown further in a glasshouse.

Outcome:

- Molecular characterization of complete whole genome sequence of *Cowpea aphid borne mosaic virus* and *Papaya ringspot virus* will helps in diversity as well as tenabling us to develop a less costly and sensitive technique for specific detection and differentiation of potyviruses co-infecting crops like cowpea and papaya.
- . Sequence diversity among isolates of CABMV and PRSV helps in understanding the viral origins, development, dispersion as well as disease etiology. This information would be useful in developing effective virus disease management programmes.
- Coat protein-mediated resistance to CABMV will evaluate in *N. tabacum* L. This is significant resistance since it allows the plants to grow to maturity and produce seed.
- *Papaya ringspot virus* and *cowpea aphid borne mosaic virus* transmission studies will helps to understand the entry and replication of virus. The binary vector with GUS may also useful as a tool for *in planta* genomic study, as it can express gene without affecting plant growth and development.

Significance of work

India is agrarian country and here a huge agricultural loss occurs due to infection of virus to the crops. For the prevention of virus infection some transgenic variety will be developed which will show resistance against potyvirus. By the genetic engineering approach like pathogen derived resistance crop production can also be improved and enhanced.

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